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14. ABSTRACT
Many breast cancers overexpress the tumor angiogenesis factor VEGF (vascular endothelial growth factor). Consequently, numerous VEGF inhibitors are being studied. Some, such as bevacizumab, extend progression-free survival but have not yet been shown to extend overall survival in breast cancer patients. We are pursuing a totally different approach to targeting VEGF: rather than inhibit VEGF our goal is to convert VEGF to act as a cell death factor. Toward this aim, we created a chimeric receptor (R2Fas) composed of domains from VEGF receptor 2 fused to the intracellular domain of the Fas cell death receptor. As controls, we generated two inactive versions of R2Fas with mutations in the VEGF receptor 2 domain or the Fas domain. Replication-defective adenoviruses expressing each of the receptors were generated and used to direct expression of the receptors in human breast cancer cell lines. We found that expression of the R2Fas chimeric receptor, but not the inactive control receptors, could induce apoptosis in several human breast cancer cell lines in vitro. These studies suggest that a receptor such as R2Fas, which converts VEGF to act as a cell death factor, could yield a new and more aggressive approach to targeting overexpressed VEGF in breast cancer.

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Introduction

Many breast cancers overexpress the angiogenic growth factor VEGF (vascular endothelial growth factor; also known as vascular permeability factor) which stimulates tumor angiogenesis. In breast cancer patients, overexpression of VEGF correlates with increased metastatic potential, resistance to systemic adjuvant therapies, HER2 expression, and shorter relapse-free survival and overall survival in early breast cancer [1]. Consequently, numerous VEGF inhibitors are being developed and tested. Some, such as the neutralizing anti-VEGF antibody bevacizumab, extend progression-free survival but have not yet been shown to extend overall survival in breast cancer patients. We are pursuing a totally different and unexplored approach to targeting VEGF: rather than inhibit VEGF our goal is to convert VEGF to act as a cell death factor. Toward this aim, we created a chimeric receptor (termed R2Fas) composed of domains from VEGF receptor 2 fused to the intracellular domain of the Fas cell death receptor. We previously demonstrated that VEGF killed cells transfected with the R2Fas receptor by binding to and aggregating the receptor, which activates the intracellular Fas domain to initiate apoptotic signaling [2]. In this grant we hypothesized that expression of the R2Fas receptor in human breast cancer cells that overexpress VEGF would cause them to undergo apoptosis. As controls, we generated two inactive versions of R2Fas with either a deletion in the VEGF receptor 2 domain that would abolish VEGF binding or a point mutation in the Fas domain that would abolish initiation of apoptotic signaling. If cells are killed by R2Fas but not by the control receptors, it would prove that R2Fas-induced killing is mediated by VEGF binding and by apoptotic signaling by the Fas domain of R2Fas. To express each receptor in human breast cancer cell lines in vitro, we generated replication-defective adenoviruses expressing each receptor gene. In this Concept Award, we investigated whether expression of the R2Fas receptor in human breast cancer cells induced apoptosis, to determine if this approach may point toward a new strategy for targeting VEGF in breast cancer.

Body

Task 1. Express the artificial death receptor R2Fas in human breast cancer cell lines in vitro.

Human breast cancer cell lines were obtained from the Cell Culture repository or from colleagues at UCSF and grown in vitro using established cell culture media and conditions. The cDNA encoding the R2Fas receptor was generated by ligating a DNA fragment encoding the extracellular and transmembrane domains of mouse VEGF receptor 2 (amino acids 1 to 787) to a DNA fragment encoding the complete cytoplasmic domain of human Fas receptor (amino acids 191 to 344). ΔR2Fas is an inactive control receptor with a deletion of domain 2 from the extracellular domain of VEGFR2. Domain 2 is the domain at which VEGF binds to VEGFR2, so by deleting that VEGF-binding domain we created a control receptor that we hypothesized would not bind VEGF and would not be activated by VEGF. The ΔR2Fas control receptor would be used to demonstrate that simple expression of a Fas-containing transgene would not by itself stimulate apoptosis. The cDNA encoding ΔR2Fas was generated by deleting the BsrG1-EcoRV fragment from the R2Fas plasmid and religating after Klenow

treatment to restore the reading frame. CG-R2Fas is an inactive control receptor with a previously described point mutation in the C-terminus of the Fas domain that abolishes Fas apoptotic signaling [3]. The CG-R2Fas control receptor would be used to demonstrate that apoptosis stimulated by R2Fas requires a functional Fas domain. The cDNA encoding CG-R2Fas was generated by subcloning an existing DNA fragment with the C-to-G point mutation into R2Fas [2]. A hemagglutinin epitope tag was included on the N-terminus of all three receptors to allow detection by immunoblot using anti-HA antibody.

To generate replication-defective adenoviruses expressing R2Fas, ΔR2Fas, or CG-R2Fas we used the Adeno-X reagents from Clontech. In the first step, the cDNAs encoding R2Fas, ΔR2Fas, or CG-R2Fas were subcloned into the pShuttle vector using XbaI and BamHI sites. The resulting vectors were propagated, purified, digested with I-Ceu I and I-Sce-1 and ligated to the predigested I-Ceu I + I-Sce-1 adenoviral vector purchased from the vendor. The resulting AdV/R2Fas, AdV/ΔR2Fas, and AdV/CG-R2Fas plasmids were propagated, purified using Qiagen columns, linearized by Pac I digestion, and transfected into 293 cells using a calcium phosphate protocol. When cytopathic plaques were first apparent in ~10 days, virus was recovered from freeze/thaw lysed cells. Viral expression by 293 cells was amplified by several rounds of reinfection. To purify the final amplified adenoviral preparation we used the Adeno-X purification kit from Clontech that uses an adenoviral affinity column with elution buffer. To determine the adenoviral titer we used the Adeno-X Titer kit from Clontech with titers determined using 293 cells. The final titers that we obtained over multiple preparations were 1 - 5 X 10^9 virions/mL for AdV/R2Fas, 0.2 X 10^9 virions/mL for AdV/ΔR2Fas, and 1 - 5 X 10^9 virions/mL for AdV/CG-R2Fas. These titers are in the expected range for the Adeno-X reagents. We encountered multiple technical challenges working our way through these protocols to generate purified adenovirus and the process took longer than anticipated but in the end we succeeded.

To determine if the purified AdV/R2Fas was functional we infected T47D human breast cancer cells in vitro with different multiplicities-of-infection (ratio of virions/cell) and performed immunoblot analysis 72 hours after infection. As shown in Figure 1, we found that expression of R2Fas was detectable at an MOI=5 and was highly expressed at an MOI=40. Therefore we used an MOI=40 for subsequent experiments. MOI>100 can in some cases introduce artifactual cytotoxic responses. To determine the time course of R2Fas expression after AdV/R2Fas infection, we performed a time course experiment (Figure 2). T47D cells were infected with AdV/R2Fas and at various times (18 – 96 hours) after infection cells were lysed and immunoblot analysis performed. As seen in Figure 2, R2Fas expression was detectable as early as 18 hours after infection and only gradually increased from 48 hours to 96 hours after infection. Therefore we used 48 to 72 hour timepoints for subsequent experiments.

T47D breast cancer cells express VEGF and our hypothesis was that if R2Fas were expressed in these cells, the VEGF they secrete would activate R2Fas to induce apoptotic signaling and cell death. As seen in Figure 3, when T47D cells were infected with AdV/R2Fas, expression of R2Fas was detectable beginning at 12 hours and increasing out to 48 hours. At those time points we also saw activation of apoptotic signaling, as determined by immunoblot detection of cleaved (activated) caspase-3 and cleavage of PARP. Importantly, the control cells received the control virus AdV/LacZ

also at an MOI=40, and did not show cleavage of caspase-3 or PARP, demonstrating that adenoviral infection itself does not induce apoptotic signaling. These data demonstrated that expression of R2Fas stimulates apoptotic signaling in T47D cells.

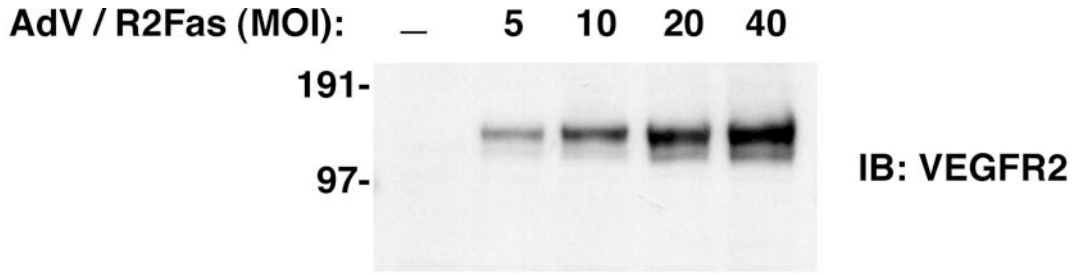

Figure 1. Titer-dependent adenoviral-mediated expression of R2Fas. T47D human breast cancer cells grown in 6-well plates were infected with AdV/R2Fas at various multiplicities-of-infection (MOI). Seventy-two hours after infection cells were lysed and lysates were subjected to SDS-PAGE. Expression of R2Fas was visualized by immunoblotting with an anti-VEGFR2 antibody that recognizes the extracellular domain of VEGFR2 that is present in R2Fas.

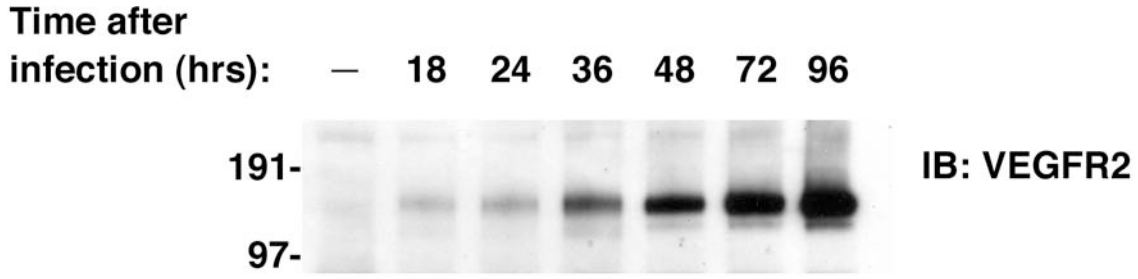

Figure 2. Time-dependent adenoviral-mediated expression of R2Fas. T47D human breast cancer cells were infected with AdV/R2Fas at an MOI=40. At the indicated times after infection cells were lysed and lysates were subjected to SDS-PAGE. Expression of R2Fas was visualized by immunoblotting with an anti-VEGFR2 antibody.

We performed a similar experiment using MCF7 breast cancer cells (Figure 4). In this experiment we included in one well the caspase inhibitor ZVAD-fmk. We again found that expression of R2Fas induced caspase-3 activation, which could be blocked by addition of ZVAD, confirming that R2Fas activated caspase-mediated signaling. We also performed a cytotoxicity assay (LDH release, Roche) and found a titer-dependent cytotoxic response to R2Fas expression that was blocked by addition of ZVAD.

In contrast to T47D and MCF7 cells, when we performed a similar experiment in MDA-MB-231 breast cancer cells, which are known to be resistant to Fas-mediated signaling, we found that while R2Fas was expressed there was no activation of caspase-3 (Figure 5).

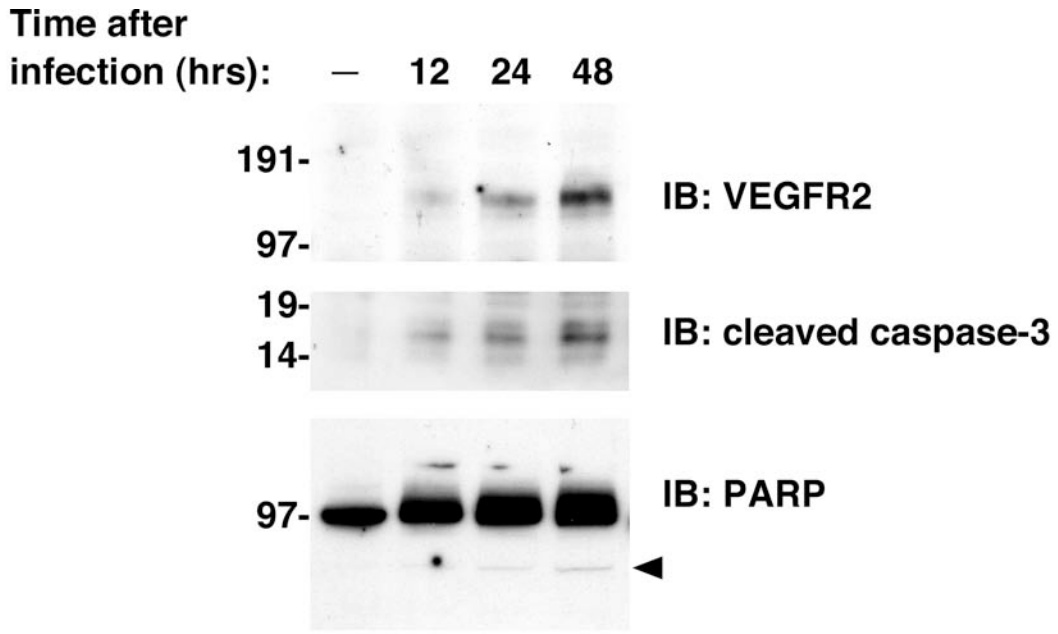

Figure 3. Adenoviral-mediated expression of R2Fas in T47D human breast cancer cells activates apoptotic signaling . T47D human breast cancer cells were infected with AdV/R2Fas at an MOI=40. At the indicated times after infection cells were lysed and lysates were subjected to SDS-PAGE. Expression of R2Fas, cleaved/activated caspase-3, and cleaved PARP (arrowhead) were visualized by immunoblotting.

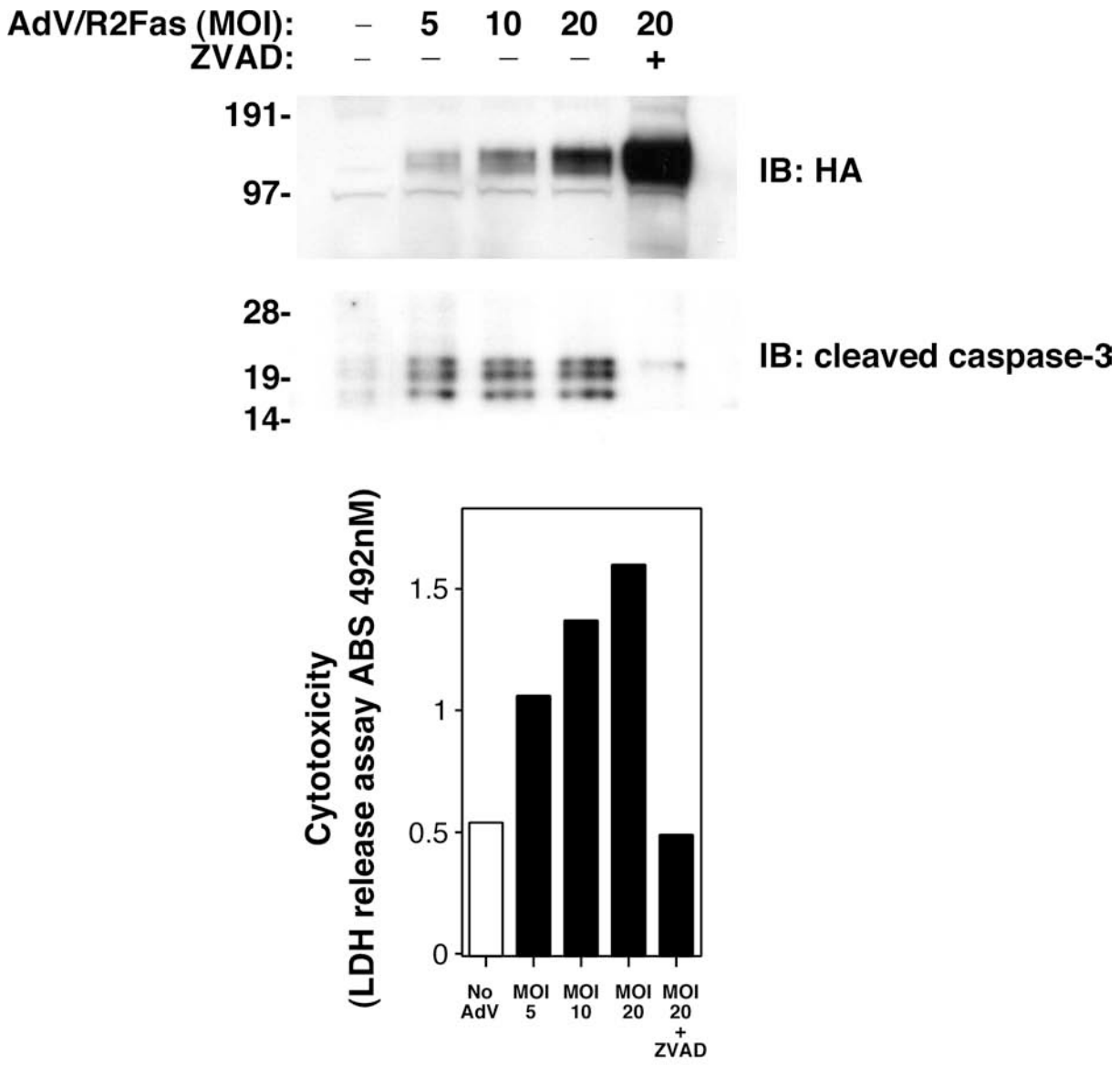

Figure 4. Adenoviral-mediated expression of R2Fas in MCF7 human breast cancer cells activates apoptotic signaling and cytotoxicity that is blocked by ZVAD (Top) MCF7 human breast cancer cells were infected with AdV/R2Fas at the indicated MOI, in the absence or presence of the caspase inhibitor ZVAD. Forty-eight hours after infection cells were lysed and lysates were subjected to SDS-PAGE. Expression of R2Fas was visualized by immunoblotting with an anti-HA tag antibody, and activated caspase-3 with a cleaved caspase-3-specific antibody. ZVAD inhibited caspase-3 activation. **(Bottom)** MCF7 cells were infected with AdV/R2Fas at the indicated MOI in the absence or presence of the caspase inhibitor ZVAD. Forty-eight hours after infection cytotoxicity was assayed using the LDH release assay (Roche). ZVAD blocked R2Fas-induced cell death.

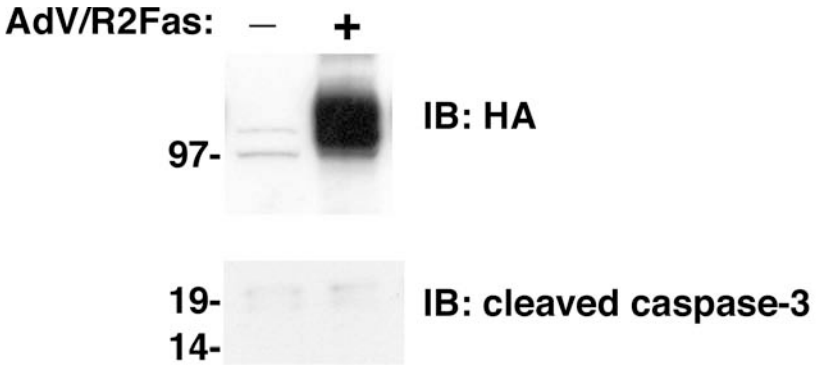

Figure 5. Adenoviral-mediated expression of R2Fas in MDA-MB-231 human breast cancer cells does not activate apoptotic signaling. MDA-MB-231 human breast cancer cells were infected with AdV/R2Fas at MOI=40. Seventy-two hours after infection cells were lysed and lysates were subjected to SDS-PAGE. Expression of R2Fas was visualized by immunoblotting with an anti-HA tag antibody, and activated caspase-3 with a cleaved caspase-3-specific antibody.

Task 2.Determine if R2Fas expression stimulates apoptosis in breast cancer cells.

Apoptosis is characterized morphologically by membrane blebs, cytoplasmic swelling, and ultimately cell fragmentation. These changes were readily detectable after R2Fas expression in T47D and MCF7 cells but not in MDA-MB-231 cells. Figures 6-8 show these cells after infection with AdV/R2Fas or the control LacZ virus. Both T47D and MCF7 cells demonstrated cell fragmentation and cell loss.

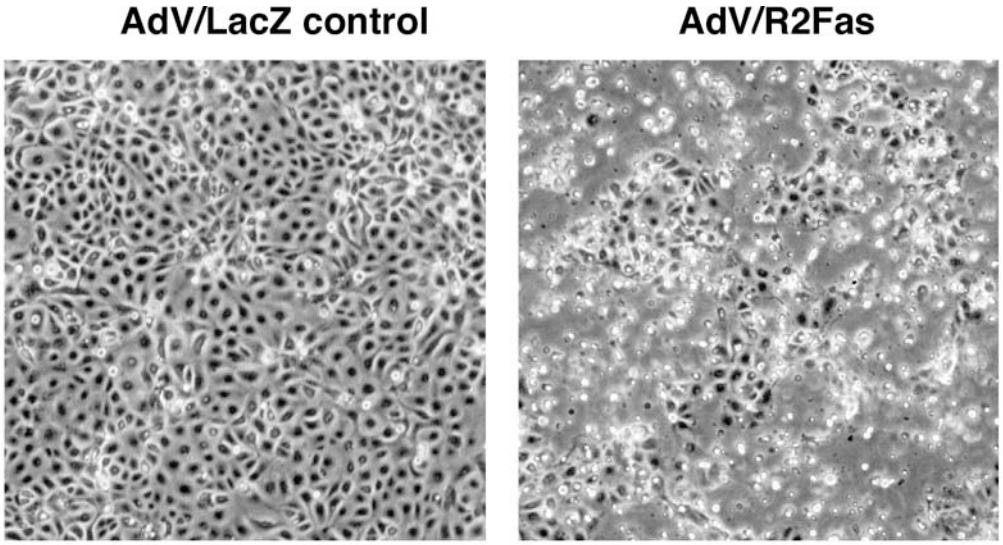

Figure 6. Expression of R2Fas induces cell fragmentation, membrane blebbing, and cell death in T47D cells. T47D human breast cancer cells were infected with AdV/R2Fas or the control virus AdV/LacZ at an MOI-40. Seventy-two hours after infection cells were photographed.

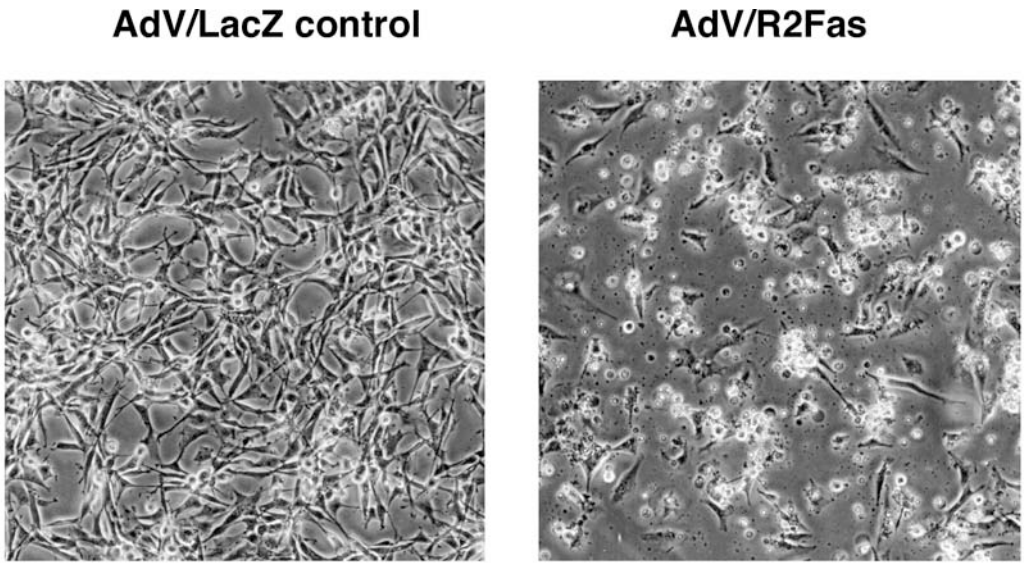

Figure 7. Expression of R2Fas induces cell fragmentation, membrane blebbing, and cell death in MCF7 cells. MCF7 human breast cancer cells were infected with AdV/R2Fas or the control virus AdV/LacZ at an MOI-40. Seventy-two hours after infection cells were photographed.

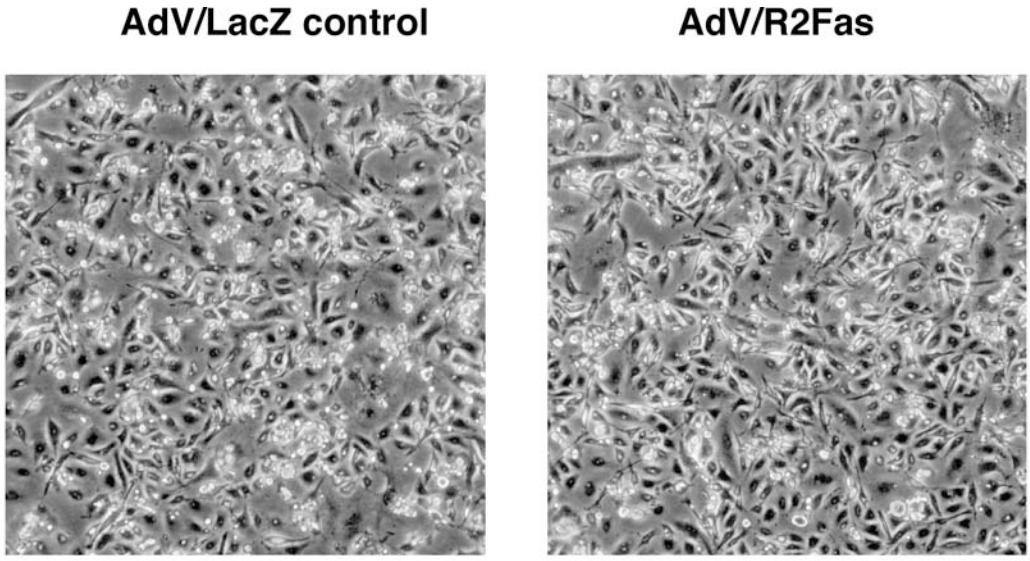

Figure 8. Expression of R2Fas does not induce cell fragmentation, membrane blebbing, and cell death in MDA-MB-231 cells. MDA-MB-231 human breast cancer cells were infected with AdV/R2Fas or the control virus AdV/LacZ at an MOI-40. Seventy-two hours after infection cells were photographed.

To quantify R2Fas-induced cytotoxicity, we performed the LDH release assay on the three cell lines after AdV/R2Fas or control LacZ virus infection (Figure 9).

Consistent with the immunoblot analysis showing caspase activation and the morphologic changes seen, we found that R2Fas expression induced a cytotoxic response in T47D and MCF7 cells, but not in MDA-MB231 cells.

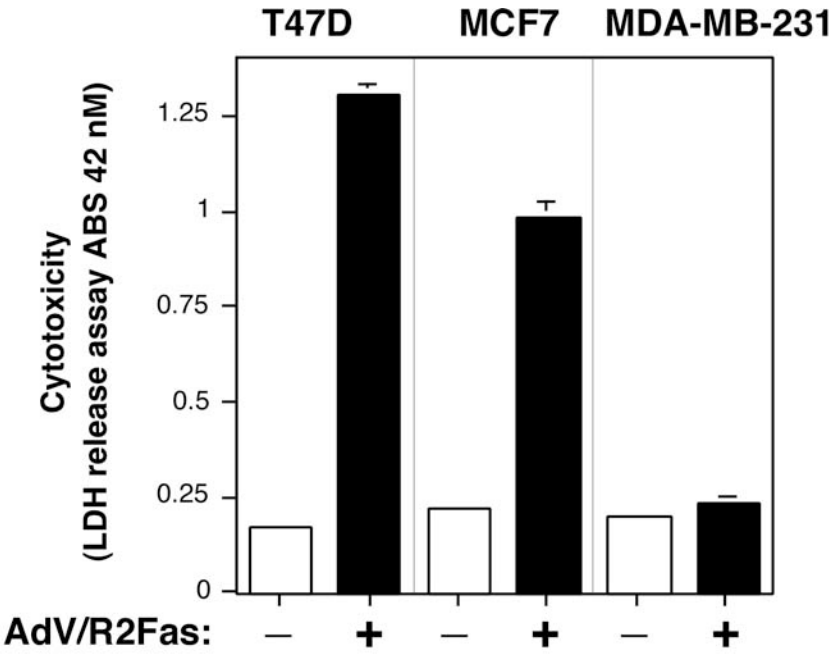

Figure 9. Expression of R2Fas induces cell death in T47D and MCF-7 but not MDA-MB-231 human breast cancer cells. T47D, MCF-7, and MDA-MB-231 cells were grown in 6-well plates and infected with AdV/R2Fas at an MOI=40 or with AdV/Lac Z as control at the same MOI. Forty-eight hours after infection cytotoxicity was assayed by quantifying LDH release into the supernatant (Roche).

Task 3. Exclude non-specific mechanisms of apoptosis by using control adenoviruses.

These data confirmed our hypothesis that expression of R2Fas could induce apoptosis in some breast cancer cell lines, and the absence of apoptosis in cells infected with the control LacZ virus demonstrated that apoptosis is specifically induced by R2Fas expression. To more rigorously demonstrate that R2Fas induces apoptosis by the intended mechanism (secreted VEGF binds to R2Fas which then activates the Fas receptor) we examined the response of cells to expression of ΔR2Fas and CG-R2Fas. As seen in Figures 10 – 12, infection of the three cell lines with AdV expressing R2Fas, ΔR2Fas, or CG-R2Fas produced high levels of receptor expression.

T47D cells and MCF7 cells expressing R2Fas again showed apoptotic cell death, but in those two cell lines there was no apoptotic response to ΔR2Fas or CG-R2Fas expression. This result is important proof of the mechanism of action of R2Fas. Apoptosis induced by R2Fas but not by ΔR2Fas, the mutant receptor with a deletion of the VEGF-binding domain, demonstrates that binding of VEGF to R2Fas is required for

apoptosis. Similarly, apoptosis induced by R2Fas but not by CG-R2Fas, the mutant receptor that can bind VEGF but cannot initiate apoptotic signaling, demonstrates that R2Fas-induced apoptosis is mediated by its Fas domain. As expected, MDA-MB-231 cells had no response to any receptor.

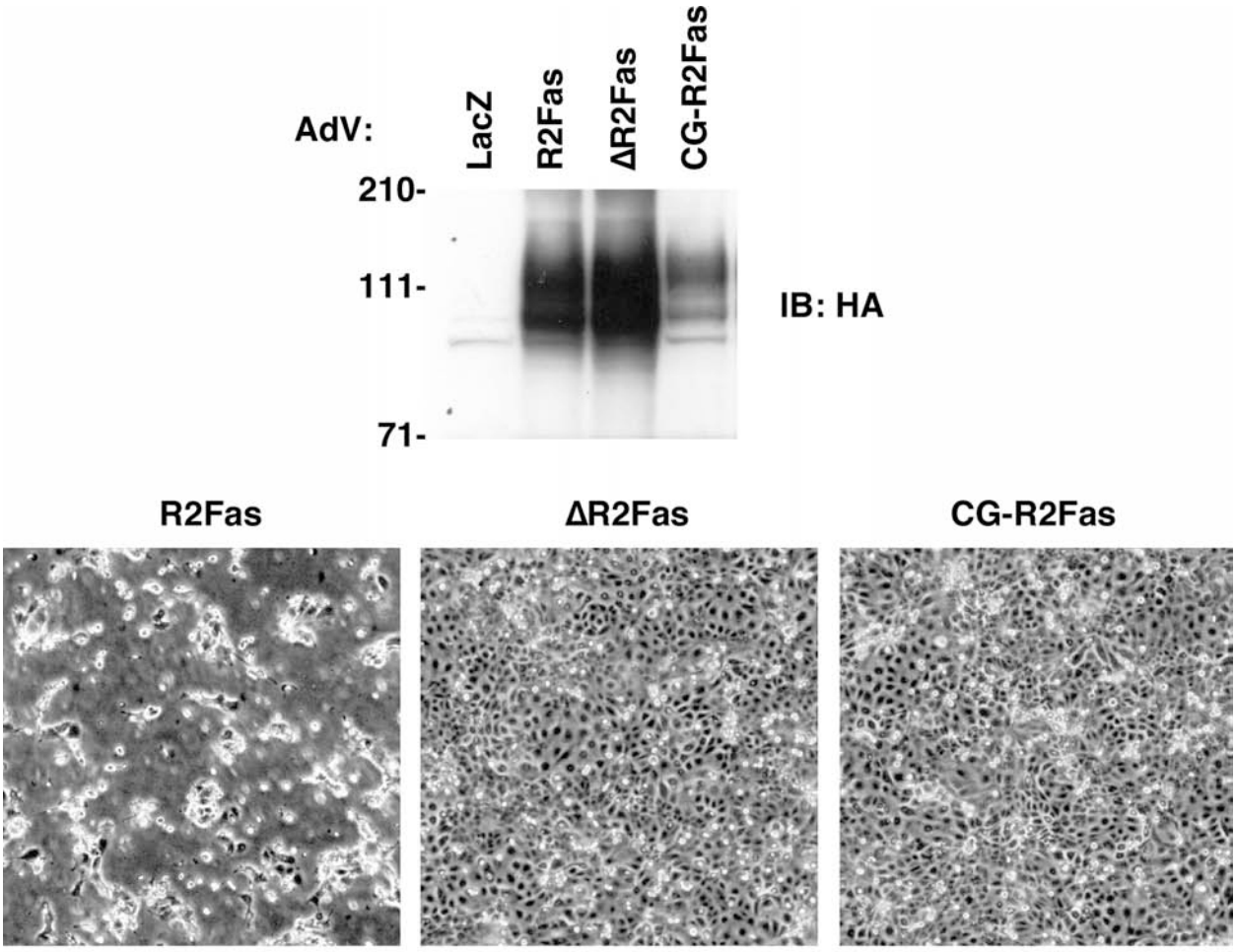

Figure 10. Expression of R2Fas but not ΔR2Fas or CG-R2Fas stimulates cell death in T47D human breast cancer cells. (Top) T47D cells were infected with AdV/R2Fas, AdV/ΔR2Fas, AdV/CG-R2Fas, or the control virus AdV/LacZ at an MOI=40. Forty-eight hours after infection cells were lysed and expression of the receptors was determined by SDS/PAGE and anti-HA immunoblot. (Bottom) In a parallel experiment infected cells were photographed at seventy-two hours.

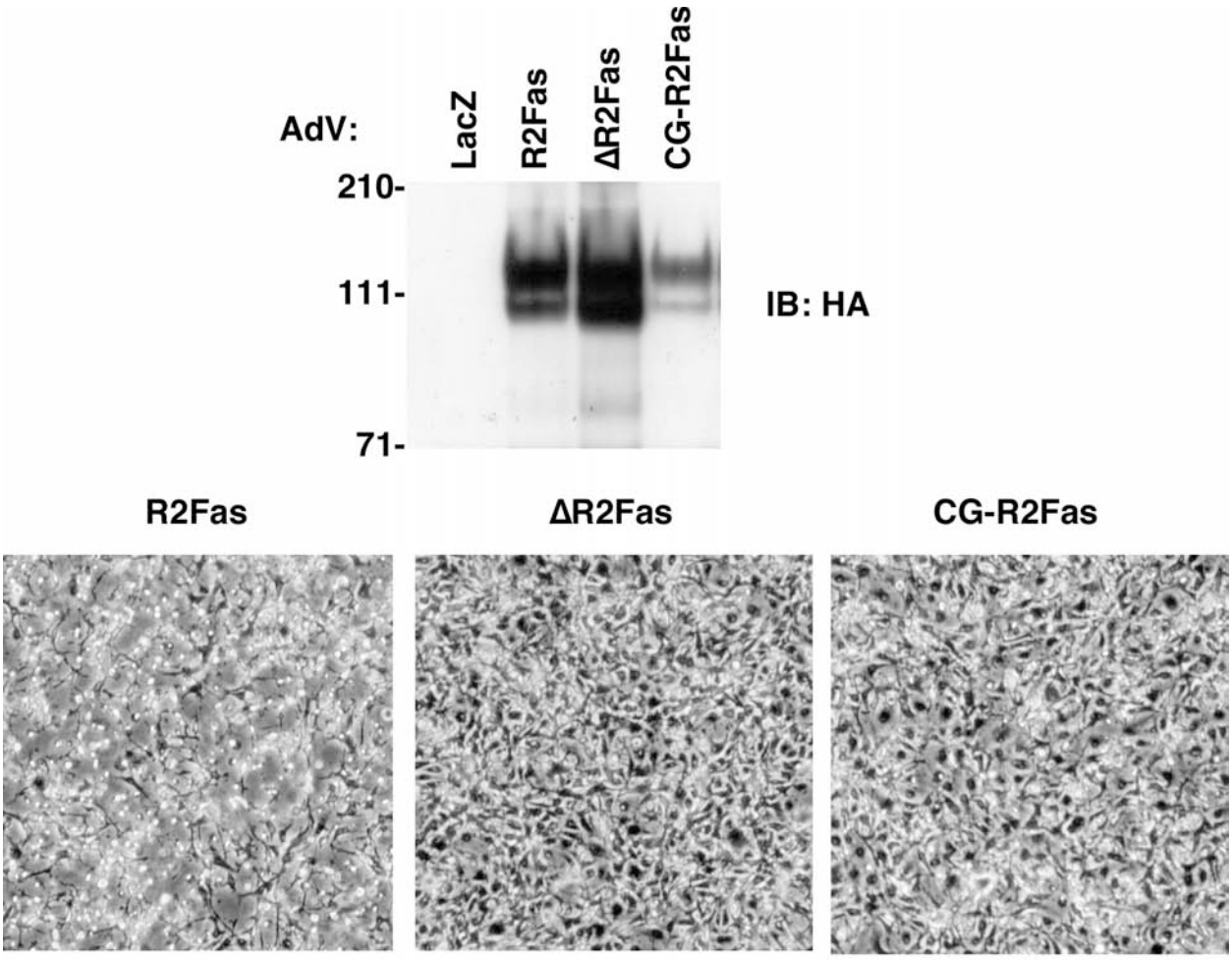

Figure 11. Expression of R2Fas but not ΔR2Fas or CG-R2Fas stimulates cell death in MCF7 human breast cancer cells. (Top) MCF7 cells were infected with AdV/R2Fas, AdV/ΔR2Fas, AdV/CG-R2Fas, or the control virus AdV/LacZ at an MOI=40. Forty-eight hours after infection cells were lysed and expression of the receptors was determined by SDS/PAGE and anti-HA immunoblot. (Bottom) In a parallel experiment infected cells were photographed at seventy-two hours.

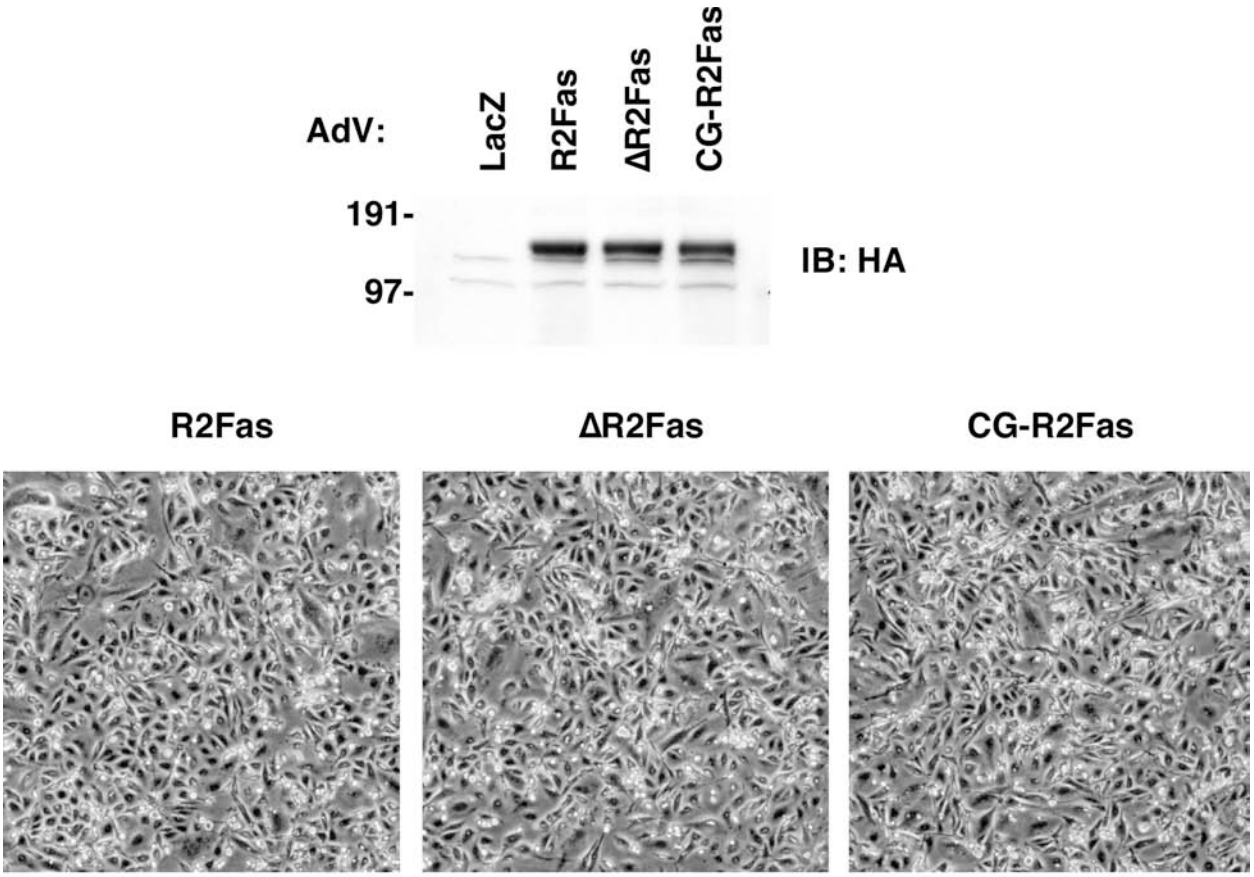

Figure 12. Expression of R2Fas, ΔR2Fas or CG-R2Fas does not stimulate cell death in MDA-MB-231 human breast cancer cells. (Top) MDA-MB-231 cells were infected with AdV/R2Fas, AdV/ΔR2Fas, AdV/CG-R2Fas, or the control virus AdV/LacZ at an MOI=40. Forty-eight hours after infection cells were lysed and expression of the receptors was determined by SDS/PAGE and anti-HA immunoblot. **(Bottom)** In a parallel experiment infected cells were photographed at seventy-two hours.

Task 4. Determine if normal breast epithelial cells express low levels of VEGF and are resistant to being killed by R2Fas expression.

The purpose of this task was to determine if R2Fas showed specificity for killing breast cancer cells that express VEGF and did not kill normal primary breast epithelial cells. We were not able to answer this question. The normal primary breast epithelial cells grew poorly and stopped proliferating after only several passages. At a cost of $600 for a single vial of cells plus medium (Clonetics), it was not wise or affordable to keep buying more vials of cells. For several reasons this doesn't lessen the value of the preceding data demonstrating that R2Fas induces apoptosis in breast cancer cells. If R2Fas is tested in a preclinical animal model of breast cancer it will be delivered by direct intratumoral injection into a subcutaneous xenograft, and therefore will have little expression in normal breast tissue. If R2Fas advanced toward clinical use in a gene therapy trial, like most potentially cytotoxic proteins its expression would likely be regulated by a combination of viral transduction targeting and transcriptional regulation.

Key Research Accomplishments

1. Generated cDNAs encoding the R2Fas, ΔR2Fas, and CG-R2Fas chimeric receptors.
2. Generated replication-defective adenoviruses expressing the R2Fas, ΔR2Fas, and CG-R2Fas receptors.
3. Demonstrated adenoviral-mediated expression of the R2Fas, ΔR2Fas, and CG-R2Fas receptors in human breast cancer cell lines in vitro.
4. Demonstrated that expression of R2Fas, but not the control receptors, stimulated apoptosis in T47D and MCF7 human breast cancer cell lines.
5. Established the feasibility of converting overexpressed VEGF to act as an autocrine cell death factor in breast cancer cells.

Reportable outcomes

1. An abstract and poster describing this strategy was presented at the American Association for Cancer Research Annual Meeting, April 2005 in Anaheim.

2. A patent titled "VEGF-activated Fas Ligands" was filed by the University of California on 8/15/06, International Application Number: PCT/US2006/031991. Support from this grant is acknowledged on page 2 of the application, which is included in the appendix. The patent runs 105 pages and is 10 MB, so only the first five pages of the patent are included.

3. A manuscript describing this work is in preparation.

4. The replication-defective adenoviruses expressing the R2Fas chimeric death receptor and the inactive control receptors ΔR2Fas and CG-R2Fas will be shared with other interested investigators.

5. Grants applied for based on work supported by this Concept Award:
   Accelerate Brain Cancer Cure Foundation, March 2005
   Ovarian Cancer Research Program, DOD, February 2005
   Prostate Cancer Foundation grant, awarded January 2006
   Prostate Cancer Foundation grant, awarded January 2007
   Breast Cancer Research Program, Univ. of California, January 2007
   Tobacco-Related Disease Research Program, Univ. of Cal., awarded July 2007

Conclusion

In this Concept Award our goal was to determine if the novel chimeric R2Fas death receptor, which triggers apoptosis in the presence of VEGF, induces apoptosis in human breast cancer cell lines. We demonstrated that human breast cancer cells in vitro can be adenovirally transfected to express the R2Fas receptor, and demonstrated that in some cell lines R2Fas expression stimulated apoptotic signaling and induced apoptosis. This, and our previous work on prostate cancer cells, appears to be the first

demonstration that a growth factor secreted by a cancer cell can be forced to act as an autocrine cell death factor by activating an engineered receptor. These findings suggest that this strategy may be feasible to treat human cancer. A limitation to this approach is the requirement for gene transfer of the R2Fas receptor into cancer cells. This requirement for a gene therapy technology could hinder the development of this idea, because despite significant advancements in gene therapy vectors over the past fifteen years there remain many technical, safety, and potentially regulatory challenges to be overcome before gene therapy technology is safe and effective. With this caveat in mind, our demonstration that VEGF secreted by cancer cells could be made to kill those cells suggested to us a less technically complex approach to accomplish the same goal, which is to create a recombinant fusion protein composed of a VEGF-binding domain fused to Fas ligand or TRAIL ligand. We have successfully begun to pursue that strategy, which arose from the studies funded by this grant.

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3. Nagata, S. Mutations in the Fas antigen gene in lpr mice. Semin Immunol, 6:3, 1994.

Appendices

1. Patent application.
   Title: “VEGF-activated Fas Ligands”
   File date: 8/15/06
   International Application Number: PCT/US2006/031991
   Inventor: Timothy P. Quinn
   Assignee: University of California
   Support from the BCRP acknowledged in the patent on page 2.

Bibliography

Abstract: “Killing cancer cells and endothelial cells with a VEGF-triggered cell death receptor”, Quinn TP, San Mateo C and Padron A, American Association for Cancer Research Annual Meeting, April 2005 in Anaheim.

Personnel receiving salary:
PI: Timothy P. Quinn, M.D.